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(54) Title: CYTOTOXINS SPECIFIC FOR GM-CSF RECEPTOR-BEARING CELLS

(57) Abstract

Conjugates containing granulocyte-macrophage colony-stimulating factor (GM-CSF) conjugated to a toxic molecule are provided. The conjugates are used for ameliorating GM-CSF-mediated or GM-CSF-associated disorders, including cancers and inflammatory disease. In a preferred embodiment, GM-CSF is chemically conjugated by disulfide bonds to the ribosome-inactivating protein saporin. The resulting conjugate is highly toxic to targeted cells.

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CYTOTOXINS SPECIFIC FOR GM-CSF RECEPTOR-BEARING CELLS

This application is a continuation-in-part of United States Serial No. 07/898,816, filed June 15, 1992, by Douglas A. Lappi and J. Andrew Baird, entitled "MITOTOXINS SPECIFIC FOR GM-CSF RECEPTOR-CONTAINING CELLS". The disclosure of United States Serial No. 07/898,816 is incorporated herein by reference thereto.

FIELD OF THE INVENTION

This invention relates to cytotoxic conjugates containing a lymphokine attached to a toxic molecule. The conjugates are useful for targeting toxins to cells that are implicated in a variety of pathological conditions. In particular, conjugates containing granulocyte-macrophage colony-stimulating factor coupled to a toxic molecule are provided.

BACKGROUND

The differentiation, activation, and proliferation of the various blood cells arising from multi-potential stem cells are controlled by a family of glycoproteins synthesized by multiple cell types distributed in many tissues (see, e.g., Schrimsher et al. (1987) Biochem. J. 247:195-199).

One such glycoprotein, granulocyte-macrophage colony-stimulating factor (GM-CSF), is produced by a variety of cell types in response to specific signals, such as T cells, macrophages, mast cells, endothelial cells, and fibroblasts. GM-CSFs from mammalian sources have been isolated and characterized. Human GM-CSF (hGM-CSF) is a glycoprotein 144 amino acids in length, including a 17 amino acid leader sequence, with an apparent molecular weight of approximately 22,000 daltons. Murine GM-CSF, which is also glycosylated, is 141 amino acids in length and has a 17 amino acid leader sequence. Two intrachain disulfide bonds, which are highly conserved and important for biological activity, are found in murine and hGM-CSF.

Numerous biological activities have been attributed to GM-CSF, which is thought to have its primary role in regulation of hematopoiesis.

GM-CSF stimulates the proliferation and maturation of myeloid progenitor cells, which mature to neutrophilic and eosinophilic granulocytes and monocytes. GM-CSF is also reported to stimulate the proliferation of endothelial cells, and enhance the function of mature circulating cells such as neutrophils, eosinophils, basophils, macrophages and other cell types (see, e.g., Gasson (1991) Blood 77:1131-1145, 1135 (Table II)). GM-CSF is also known to induce endothelial cells to migrate and proliferate. Recombinant GM-CSF has been used to stimulate the migration of human endothelial cell lines, and increase the incorporation of radioisotope-labelled thymidine into DNA (see, Bussolino et al. (1989) Nature 337, 471-473).

GM-CSF acts upon cells through binding with a specific cell-surface receptor that is composed of at least two subunits. The α subunit, a mature protein of approximately 80,000 daltons, binds GM-CSF with a low affinity. The β subunit does not bind GM-CSF, but the α and β subunits form a high affinity receptor complex that mediates signal transduction. The Kd of the high affinity (α plus β) receptor for GM-CSF is approximately 40 pM, but varies from 10 to 40 pmol/L depending on the type of cell studied (see, e.g., Gasson (1991) Blood 77:1131-1145, p. 1136). Low affinity receptors (e.g., α alone) have a Kd of approximately 1 nmole/L (see, e.g., Gasson (1991) Blood 77:1131-1145, 1136).

DNA encoding the α and β subunits has been cloned (see, e.g., Kitamura et al. (1991) Proc. Natl. Acad. Sci. USA 88:5082-5086); and Gearing et al. (1989) EMBO J. 8:3667-3676). DNA encoding human GM-CSF α and β subunits has been expressed in CTLL 2 cells, an IL-2-dependent mouse T cell line (see, Kitamura et al. (1991) Proc. Natl. Acad. Sci. USA 88:5082-5086).

GM-CSF and GM-CSF cell surface receptors are implicated in certain pathological conditions. GM-CSF stimulates the proliferation of

leukemic cells in culture. Human GM-CSF stimulates KG-1 myeloid leukemia cell lines to increase RNA synthesis, and subsequently DNA and protein synthesis (Lusis et al. (1980) Proc. Natl. Acad. Sci. USA 77: 5346-5350). Acute myeloid leukemia cells (AML) express GM-CSF receptors and are responsive to GM-CSF. There is also evidence that, GM-CSF is expressed in myeloid leukemias and certain cell lines, as well as by certain solid tumors, suggesting that GM-CSF acts via an autocrine mechanism in these cells, which express GM-CSF receptors (see, e.g., Gasson (1991) Blood 77:1131-1145, 1133).

GM-CSF receptors have also been located on nonhematopoietic cells. For example, high-affinity GM-CSF receptors have been identified on small-cell carcinoma of the lung (SCCL) (Baldwin et al. ((1989) Blood 73:1033). Human GM-CSF was reported to stimulate proliferation of two osteogenic sarcoma cell lines, a breast carcinoma cell line, and an SV40 transformed marrow stromal cell line. (Dedhar et al. (1988) Proc. Natl. Acad. Sci. USA 85:9253). Colon adenocarcinoma cell lines are reported to grow in response to hGM-CSF (Berdel et al. (1989) Blood 73:80).

GM-CSF has also been found in the synovial fluids from patients having inflammatory arthropathies such as rheumatoid arthritis. For example, synovial fluids from patients with classical rheumatoid arthritis, psoriatic arthritis, Rieter's syndrome, ankylosing spondylitis, and osteoarthritis and from cultured rheumatoid arthritis synovial tissues, exhibited GM-CSF activity in an in vitro cell assay. The fluids and supernatants were tested on cultures of human peripheral blood macrophage-depleted non-T cells. The cultures formed cell aggregates in response to the fluids and supernatants in a response similar to that of the recombinant GM-CSF. This response was reversed by the addition of anti-GM-CSF antibodies.

Since GM-CSF is implicated in numerous diseases, there is a need to understanding its role in the pathology of these disease states and to

develop methods and means for selectively inhibiting its activity. Therefore, it is an object herein to provide a therapeutic agent for treating disorders in which GM-CSF plays a role and to provide a tool for related studies of GM-CSF function in such pathologies.

SUMMARY OF THE INVENTION

Cytotoxin conjugates containing GM-CSF conjugated to a cytotoxic molecule are provided. The cytotoxic molecules include any molecule that is cytotoxic, and are preferably ribosome inactivating proteins, such as saporin and the ricin A chain. The cytotoxin conjugates, which can be prepared by chemically conjugating GM-CSF, are effective for inhibiting the growth and proliferation of cells that express a GM-CSF receptor. These cytotoxin conjugates are highly toxic to cells expressing GM-CSF receptors.

Preferred conjugates are those which by virtue of the linkage between the GM-CSF moiety and the toxic moiety are substantially less toxic than the unconjugated toxic moiety as assessed by cell-free assays.

In a preferred embodiment, a conjugate containing equimolar amounts of GM-CSF and the ribosome-inactivating protein saporin (SAP) chemically attached by disulfide bonding is provided. Until internalized by targeted cells that express GM-CSF receptors, this particular conjugate exhibits relatively low toxicity. Its protein synthesis-inhibiting activity is only about 10% of the activity of the saporin moiety in an unconjugated form. Once internalized by targeted cells, however, this GM-CSF-SAP conjugate is highly toxic. The GM-CSF-SAP conjugate does not affect cells that do not express the receptor.

The GM-CSF-SAP conjugate is, thus, a highly effective cytotoxin that targets only cells that express GM-CSF receptors. The conjugate, thus, should be therapeutically useful for treating GM-CSF-mediated or GM-CSF-associated disorders.

Pharmaceutical compositions containg the GM-CSF conjugates and methods for treating GM-CSF-mediated or GM-CSF-associated disorders are also provided herein.

Assays for detecting GM-CSF receptors are also provided.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows the effect on protein synthesis in cell-free lysates when exposed to unreduced GM-CSF-SAP (■), reduced GM-CSF-SAP (●) free SAP (▵), and free GM-CSF (□).

FIG. 2a shows the effect on thymidine incorporation in CTLL$\alpha\beta$-4/GM cells which express GM-CSF receptors when exposed to GM-CSF-SAP (●), free GM-CSF (□), SAP (▵), and a mixture of GM-CSF and SAP (○). FIG 2b shows the effect on cell number of CTLL$\alpha\beta$-4/GM cells when exposed to GM-CSF-SAP (●), free GM-CSF (□), free SAP (▵), and an equimolar mixture of GM-CSF and SAP (○). FIG 2c shows the effect of GM-CSF-SAP on thymidine incorporation for CTLL$\alpha\beta$-4/GM cells which express GM-CSF receptors (●), and CTLL-2 cells lines which do not express GM-CSF receptors (○).

FIG. 3a shows the effect on thymidine incorporation in CTLL cell line AIC2Bα-3 when exposed to .1 nM (100 pm) GM-CSF-SAP, and increasing concentrations of GM-CSF. AIC2Bα-3 cells express the GM-CSF receptor. FIG. 3b shows the effect on thymidine incorporation in CTLL AIC2Bα-3 cells when exposed to 200 pM GM-CSF-SAP and the indicated growth factors.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

Definitions

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as is commonly understood by one of skill in the art to which this invention belongs. All patents and publications referred to herein are incorporated by reference thereto.

As used herein, mitotoxin refers to a conjugate between the cytotoxic molecule and a mitogen.

As used herein, a cytotoxin conjugate refers to conjugates that are produced by coupling a toxic molecule or moiety with a targeting molecule that specifically interacts with cells that bear receptors with which the molecule specifically interacts and by which the targeted conjugate is internalized. The cytotoxic molecule and targeting molecule are by any suitable means, and are preferably linked via covalent bonds. The linkage is selected so that upon internalization by a targeted cell, the targeted cells are killed or their growth or differentiation are inhibited.

The cytotoxic conjugates herein are conjugates between cytoxic moieties and the cytokine GM-CSF. The cytokine moiety acts by specifically binding to receptors thereby targeting the toxic molecule to cells expressing the receptors.

As used herein, a pro-drug refers generally to a substance that is useful therapeutically and is provided in a form that becomes effective for the purpose intended only after being internalized by the targeted cells. For example, a pro-drug cytotoxic conjugate, or a cytotoxic conjugate in pro-drug form does not exhibit toxicity to intact cells until it binds with the targeted cells having the appropriate receptor and is taken up into the cells.

As used herein, a granulocyte-macrophage colony-stimulating factors (GM-CSF) refers to human GM-CSF and other mammalian GM-CSF proteins exhibiting GM-CSF activity mediated through binding to GM-CSF receptors. GM-CSF refers to polypeptides having amino acid sequences of native GM-CSF proteins, as well as modified sequences, having amino acid substitutions, deletions, insertions or additions of the native protein but retaining the ability to bind to GM-CSF receptors and to be internalized. Any molecule that specifically targets cells that express GM-CSF receptors and internalizes a conjugated cytotoxic moiety can be

used herein. For example, GM-CSF isolated from human tissues is a 144 amino acid protein having a molecular weight of approximately 14,650 daltons. GM-CSF encompasses proteins produced by recombinant technology in heterologous cells and synthetic proteins as well as those isolated from mammalian sources. It is understood that differences in amino acid sequences can occur among GM-CSFs of different species as well as among GM-CSFs from individual organisms or species. Reference to GM-CSFs is intended to encompass proteins isolated from natural sources as well as those made synthetically, as by recombinant means or possibly by chemical synthesis. GM-CSF also encompasses muteins of GM-CSF that possess the ability to target a cytotoxin, such as saporin, to GM-CSF-receptor bearing cells

As used herein, GM-CSF receptor encompasses receptors or receptor subunits that specifically interact with GM-CSF and transport it into the cells. GM-CSF receptor include receptors or receptor subunits isolated from mammalian tissues or expressed recombinantly in heterologous cells. GM-CSF receptor also encompasses low affinity receptors and high affinity receptors, as defined by the binding coefficient (kd). For example, high affinity receptors for monocytes, U937, and TF1 cell lines have an estimated kd of 10 to 40 pmol/L for high-affinity sites and an estimated kd of approximately 1 nmol/L for low-affinity sites (see, e.g., Chiba et al. (1990) Leukemia 4:29. GM-CSF receptor also encompasses receptors made of a combination of subunits of different cell types or species that are able to bind and internalize GM-CSF from humans or other species. For example, such recombinant receptors have been produced by transfecting CTLL cell lines with DNA encoding the α subunit of human GM-CSF receptor and DNA encoding the mouse AIC2B protein encoding the mouse low-affinity IL-3 receptor. The resulting transfected cells express a receptor that specifically interacts with human

GM-CSF. (see, e.g., Kitamura et al. (1991) Proc. Natl. Acad. Sci. USA 88:5082-5086.

As used herein, GM-CSF-mediated or GM-CSF-associated pathophysiological condition refers to a deleterious condition characterized by or caused by proliferation of cells or differentiation of cells that are responsive to GM-CSF either in its capacity as a mitogen or by virtue of its ability to induce differentiation. Such pathophysiological conditions include, but are not limited to, certain solid tumors, including melanoma leukemias, such as AML, small-cell carcinoma of the lung (SCCL), osteogenic sarcomas, disease in which hematopoietic cells are implicated, and inflammatory arthropathies, such as classical rheumatoid arthritis, psoriatic arthritis, Rieter's syndrome, ankylosing spondylitis, and osteoarthritis.

As used herein, the ribosome-inactivating proteins (RIPs) are enzymes derived from plant materials that inhibit protein synthesis in eukaryotic cells. Ribosome-inactivating proteins are of two types, type 1 and type 2. Type 1 RIPs are single polypeptide chains having a molecular weight of approximately 30,000. Type 2 RIPs, such as the toxins, ricin and abrin, have a ribosome-inactivating A chain linked by a disulfide bond to a B chain that includes a cell-binding domain. Type 1 RIPs and the A chain of type 2 RIPs are preferred herein. Ribosome-inactivating proteins are thought to act by removing a single nucleotide base from specific sequences of ribosomal RNA in eukaryotic ribosomes. This prevents the interaction between ribosomes and elongation factors, resulting in an inhibition of protein synthesis.

As used herein, saporin (abbreviated herein as SAP) refers to polypeptides having amino acid sequences found in the natural plant host Saponaria officinalis, as well as modified proteins, having amino acid substitutions, deletions, insertions or additions, which still express substantial ribosome-inactivating activity. Purified preparations of saporin

are frequently observed to include several molecular isoforms of the protein. It is understood that differences in amino acid sequences can occur in saporin from different species as well as between saporin molecules from individual organisms of the same species.

Saporin polypeptides include any of the isoforms of saporin that may be isolated from Saponaria officinalis or related species or modified form that retain cytotoxic activity. In particular, such modified saporin may be produced by modifying the DNA disclosed herein by altering one or more amino acids or deleting or inserting one or more amino acids, such as a cysteine that may render it easier to conjugate to GM-CSF or other cell surface binding proteins. Any such protein, or portion thereof, that, when conjugated to GM-CSF as described herein, exhibits cytoxicity in standard in vitro or in vivo assays within at least about an order of magnitude of the saporin conjugates described herein is contemplated for use herein.

As used herein, to target a saporin-containing protein means to direct it to a cell that expresses a selected receptor. Upon binding to the receptor the saporin-containing protein is internalized by the cell and is cytotoxic to the cell.

As used herein, the term biologically active, or reference to the biological activity of a cytotoxin-containing polypeptide or cytotoxicity of a such a polypeptide, refers to the ability of such polypeptide to inhibit protein synthesis by inactivation of ribosomes either in vivo or in vitro or to inhibit the growth of or kill cells upon internalization of the saporin-containing polypeptide by the cells. Preferred biologically active saporin polypeptides are those that are toxic to eukaryotic cells upon entering the cells. Such biological or cytotoxic activity may be assayed by any method known to those of skill in the art including, but not limited to, the in vitro assays that measure protein synthesis and in vivo assays that assess cytoxicity by measuring the effect of a test compound on cell

proliferation or on protein synthesis. Particularly preferred, however, are assays that assess cytoxicity in targeted cells.

As used herein, an effective amount of a compound for treating a particular disease, is an amount that is sufficient to ameliorate, or in some manner reduce the symptoms associated with the disease. Such amount may be administered as a single dosage or may be administered according to a regimen, whereby it is effective. The amount may cure the disease but, typically, is administered in order to ameliorate the symptoms of the disease. Typically repeated administration is required to achieve the desired amelioration of symptoms.

As used herein, pharmaceutically acceptable salts, esters or other derivatives of the compounds include any salts, esters or derivatives that may be readily prepared by those of skill in this art using known methods for such derivatization and that produce compounds that may be administered to animals or humans without substantial toxic effects and that either are pharmaceutically active or are prodrugs. For example, hydroxy groups can be esterified or etherified.

As used herein, treatment means any manner in which the symptoms of a conditions, disorder or disease are ameliorated or otherwise beneficially altered. Treatment also encompasses any pharmaceutical use of the compositions herein, such as use as contraceptive agents.

As used herein, amelioration of the symptoms of a particular disorder by administration of a particular pharmaceutical composition refers to any lessening, whether permanent or temporary, lasting or transient that can be attributed to or associated with administration of the composition.

As used herein, substantially pure means sufficiently homogeneous to appear free of readily detectable impurities as determined by standard methods of analysis, such as thin layer chromatography (TLC), gel

electrophoresis, high performance liquid chromatography (HPLC), used by those of skill in the art to assess such purity, or sufficiently pure such that further purification would not detectably alter the physical and chemical properties, such as enzymatic and biological activities, of the substance. Methods for purification of the compounds to produce substantially pure compounds are known to those of skill in the art.

As used herein, ED_{50} refers to an dosage, concentration or amount of a particular test compound that elicits a dose-dependent response at 50% of maximal expression of a particular response that is induced, provoked or potentiated by the particular test compound.

Preparation of the conjugates

The conjugates provided here are chemical conjugates that contain GM-CSF linked to a cytotoxic moiety, particularly a RIP, such as saporin or ricin A chain. A preferred mitotoxin is GM-CSF chemically attached to saporin (SAP), a type 1 ribosome-inactivating protein. This GM-CSF-SAP mitotoxin exhibits approximately 10% of the ribosome-inactivating activity of unconjugated saporin in cell-free assays. The reduction in cytotoxicity of the toxic moiety that accrues by virtue of its linkage to GM-CSF should be particularly advantageous in instances in which the toxic moiety is toxic in vivo in the absence of linkage to GM-CSF. The GM-CSF ligand portion of this GM-CSF-SAP conjugate preserves its ability to bind selectively to GM-CSF receptors on cell surfaces.

The GM-CSF-SAP conjugate is, however, extremely toxic to cells expressing GM-CSF receptors, and essentially non-toxic to cells not expressing GM-CSF receptors. This mitotoxin inhibits thymidine incorporation of certain test cells expressing GM-CSF receptors with an ED_{50} of approximately 4 pM, indicating an extremely potent toxic effect. The conjugate acts exclusively through the GM-CSF receptor therefore will be competitively inhibited by GM-CSF, but not other growth factors such as fibroblast growth factor (FGF), epidermal growth facter (EGF),

the insulin-like growth factors IGF-1 and IGF-2, and transforming growth factor-alpha (TGF-α).

GM-CSF

GM-CSF may be prepared using known methods of purification or can be purchased. GM-CSF can be purified from mammalian tissues or cell lines that have been appropriately stimulated to produce GM-CSF. For example, human lymphocytes produce colony-stimulating activity upon stimulation with phytohemagglutinin. Other potential physiological sources include fibroblasts and endothelial cells, stimulated by appropriate stimuli, such as tumor necrosis factor (TNF), interleukin-1 (IL-1), and tissue plasminogen activator (TPA) (see, e.g., Gasson (1991) Blood 77:1131-1145).

Human T cells transformed with human T-cell leukemia virus (HTLV) generates continuous T-cell lines, some of which produce GM-CSF when stimulated with lectin. GM-CSF is routinely purified from conditioned cell media from one of these cell lines, the Mo cell line. (Saxon et al.(1978) Ann. Intern. Med. 88:323; and Kalyanaranaman et al. (1982) Science 218:571). GM-CSF is also obtained by recombinant expression in heterologous cells. DNA encoding human GM-CSF has been isolated from Mo cell DNA expression libraries. Expression vectors containing this DNA have been transiently expressed in COS-1 monkey cells, and the GM-CSF purified from the growth-conditioned media (Wong et al. (1985) Science 228:10-815). GM-CSF may also be expressed in E. coli and other appropriate host cells.

Toxic moieties

Any cytotoxic molecule that can be linked to GM-CSF, such that the resulting conjugate is sufficiently stable to be targeted and internalized by the targeted cells may be used. Such cytoxic molecules include toxins that kill cells or that inhibit growth, differentiation, and/or proliferation.

Preferred toxic moieties are those that in an unconjugated state produce low toxicity or no toxicity to cells, due to an inability to penetrate cells, but that are highly toxic once internalized. Suitable toxic moieties should also have a high intrinsic activity if conferred with the ability to penetrate a target cell. Such toxic molecules may thus be specifically targeted to cells having GM-CSF receptors by the GM-CSF portion of the conjugate. Examples of suitable toxic moieties include ribosome-inactivating proteins such as ricin and saporin, Pseudomonas exotoxin, and diphtheria toxin. Any such moiety that may be conjugated to GM-CSF is suitable for use herein.

Preferred toxic molecules for use in the chemical conjugate are RIPs, in particular, type 1 RIPs, such as saporin, which have one polypeptide chain, or the toxin chain of type 2 RIPS, such as ricin. These RIPs are especially suitable as the toxic moieties of the conjugate, since they have a low toxicity or no toxicity for intact cells and are known to be stable and resistant to proteases. Type 1 RIPs include saporin, dianthin, agrostin, tritin, luffin, mormordin, and gelonin.
The presently preferred toxic molecule is saporin (SAP), which is one of the most potent type 1 RIPs known (Stirpe et al. (1986) FEBS Lettrs 195:1-8). Saporin is derived from the plant Saponaria officinalis. Several derivatives of saporin are known in the art, including SAP 5, SAP 6, and SAP 9. Saporin is obtained from plant materials, such as seeds by extraction and purification methods known in the art (see, e.g., Stirpe et al. (1983) Biochem J. 216:617-625) DNA encoding saporin for expression in E. coli is also available (see, e.g., GB 2 216 891 B to FARMITALIA and co-pending U.S. Patent Application Serial No. 07/901,718).

Preparation of GM-CSF conjugates

GM-CSF may be suitably conjugated to a toxic molecule of interest using chemical reactions known in the art, or chimeras may be prepared

by recombinant methods involving the expression of vectors including DNA encoding for the toxic moieties and GM-CSF. Methodologies for recombinant expression as applied to other conjugates are provided, for example, in Chaudhary et al. (1987) Proc. Natl. Acad. Sci. USA 84:4538-4542, and Maniatis et al.(1982), Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory; see, also, co-pending, U.S. Patent Application Serial No. 07/901,718).

An effective cytotoxic conjugate has been obtained by chemically conjugating GM-CSF to saporin by derivation with reactive sulfhydryl groups. The preferred method of coupling GM-CSF to SAP is the derivation with N-succinimidyl-3(2-pyridyldithio)-propionate (SPDP) to produce disulfide bonding between the GM-CSF and SAP. This reaction is described in detail in Example 1A. SAP and GM-CSF are derivatized with SPDP, introducing a reactive thiol to each moiety which are then reacted together. Synthesis of the conjugate is assessed by gel electrophoresis, and by following the release of pyridyl-2-thione, which absorbs at 343 nm. Unreacted GM-CSF and SAP are removed by ion exchange chromatography. The final product is analyzed by SDS gel electrophoresis. The major gel band reacts with polyvalent rabbit anti-human GM-CSF antibody and rabbit anti-SAP antisera by Western blotting after transfer of the band to nitrocellulose.

The resulting GM-CSF-SAP conjugate has a one-to-one molar ratio of GM-CSF and SAP. SDS gel electrophoresis shows the major band of the covalently linked GM-CSF-SAP running at approximately 45,000 daltons, corresponding to the combined weights of GM-CSF (14,650) and SAP (30,000). Western blotting using antibodies to hGM-CSF antibodies and anti-SAP antisera confirmed the presence of both molecules at the 45,000 dalton band.

Biological activity and methods of use of the conjugates

Studies described below have been conducted with GM-CSF-SAP. It is understood, however, that other cytotoxins, particularly other RIPs may be readily substituted for the SAP moiety in the conjugate. Since the methods rely on specific targeting by GM-CSF and internalization by GM-CSF receptors, and the results achieved with GM-CSF-SAP should exemplify the results that can be achieved for any cytotoxic GM-CSF conjugate.

Cytotoxicity studies

The GM-CSF-SAP conjugate chemically attached with disulfide bonds as described herein does not retain the ribosome-inactivating activity of unconjugated SAP in cell-free assays. The SAP moiety of this conjugate retains only approximately 10% of its protein synthesis-inhibition activity when compared to free SAP in cell-free rabbit reticulocyte lysates as described in detail in Example 2. This is in contrast to the retention of cytotoxic activity seen in other conjugates known in the art. For example, FGF-SAP conjugates (see, e.g., U.S. Patent No. 5,191,067 to Lappi et al., see, also, Lappi et al. (1989), Biochem. Biophys. Res. Comm. 160:917-923) retain saporin activity in cell free protein synthesis inhibition assays, and is only slightly (about two-fold) less active than free saporin.

The reduced protein synthesis-inhibiting activity of the GM-CSF-SAP conjugate is demonstrated in studies of protein synthesis in cell-free systems (see, Example 2). Unconjugated SAP has more than ten times the ribosome-inactivating activity as measured by inhibition of protein synthesis compared to GM-CSF-SAP. GM-CSF-SAP that has been reduced, prior to addition to the cell-free system, has comparable activity to free SAP in cell free assays that measure ribosome-inactivating activity. These studies indicate that it is necessary to reduce the disulfide bond connecting the two component proteins in order to

completely activate the SAP moiety of the GM-CSF-SAP conjugate. This activation of SAP occurs intracellularly after the conjugate binds to a GM-CSF receptor and is internalized into the cell. As is shown in subsequent cytotoxicity experiments, GM-CSF-SAP, when administered to intact cells expressing GM-CSF receptors, is approximately 10^4 times as toxic as SAP alone when administered to these cells. Therefore, the GM-CSF-SAP conjugate, while initially about ten times less active than SAP, becomes highly toxic when taken up by cells having GM-CSF receptors. Tumor cells, for example, having highly degradative lysosomal compartments, should readily process the GM-CSF-SAP to activate the SAP moiety.

The GM-CSF-SAP conjugate is highly toxic only to cells expressing the GM-CSF receptor. As demonstrated by in vitro studies using intact cells, the GM-CSF-SAP conjugate is essentially non-toxic to cells not expressing the GM-CSF receptor. The cytotoxicity of the GM-CSF-SAP conjugate to cells expressing the GM-CSF-SAP receptor is clearly demonstrated in the experiments described in Example 3. These studies employ mouse T-cell CTLL cell lines. The mouse GM-CSF receptor expressed by these cells does not respond to hGM-CSF. These cells lines may be transfected with DNA encoding the α and β subunits of hGM-CSF receptor (see, e.g., Kitamura et al. (1991) Proc. Natl. Acad. Sci. USA 88:5082-5086. These transfected cells express a large number of human GM-CSF receptors enabling the cells to respond to human GM-CSF by proliferation.

The GM-CSF-SAP conjugate inhibits thymidine incorporation in these CTLL cells expressing human GM-CSF receptors with an effective dosage (ED_{50}) of approximately 4 pM, indicating an extremely potent cytotoxic agent. This cytotoxic effect is dose-dependent. The non-conjugated mixture of GM-CSF and SAP has a cytotoxic effect only at a concentration of about 2000-fold higher. Because there is no targeting of SAP by GM-CSF when they are not chemically linked, a cytotoxic effect

is expected only by non-specific endocytosis. SAP by itself also has a cytotoxic effect only at a concentration of about ten fold higher than the equimolar mixture of GM-CSF and SAP.

Similarly, cell number of targeted cells decreases with increasing dosages of GM-CSF-SAP. The ED_{50} of assays of cell counts is 3 pM, similar to that in the thymidine incorporation assay.

Other experiments compare the effect of the GM-CSF-SAP conjugate on thymidine incorporation of CTLL cell lines expressing GM-CSF receptors with CTLL cell lines which do not express GM-CSF receptors. The conjugate effectively inhibits thymidine incorporation of cells expressing GM-CSF receptors with an ED_{50} of approximately 4 pM as expected, while there is no inhibition of thymidine incorporation for cells not expressing the receptors. This indicates that GM-CSF-SAP is specific for cells expressing the GM-CSF receptor.

It has also shown that the GM-CSF-SAP conjugate provided herein acts through the GM-CSF receptor to achieve its cytotoxic effect. Inhibition of thymidine incorporation by cells expressing GM-CSF receptors is reversed by ten to a thousand-fold excess quantities of GM-CSF but not an equimolar amount of GM-CSF. The inhibition of incorporation of thymidine of cells expressing GM-CSF receptors is reversed by a 100-fold molar excess of GM-CSF, but not by a 100-fold molar excess of other growth factors, including FGF, EGF, IGF-1 and IGF-2, and TGF-α. These results are consistent with competitive inhibition of GM-CSF-SAP by GM-CSF for the GM-CSF receptor and provide additional evidence that the GM-CSF conjugate acts through the GM-CSF receptor.

Formulation and use of pharmaceutical compositions

The GM-CSF conjugates provided herein, particularly the GM-CSF-SAP conjugate made via disulfide cross-linking, are pro-drugs that can be used to ameliorate the deleterious effects of GM-CSF-induced mitosis. Preferred conjugates useful as a pro-drug are those which by virtue of the

bonding between the GM-CSF moiety and the toxic moiety exhibit reduced activity of the toxic moiety when compared with the toxic moiety in an unconjugated state. The GM-CSF-SAP conjugate is particularly suitable for use as a pro-drug because of the lack of toxicity of the SAP moiety when conjugated to GM-CSF with disulfide bonding. The GM-CSF-SAP conjugate is also particularly suitable as a pro-drug because it is an extremely potent cytotoxin when acting specifically through binding with the GM-CSF receptor.

The pro-drug conjugates herein are used to treat GM-CSF-mediated pathophysiological conditions by targeting and killing or inhibiting the growth of cells and tissues that express GM-CSF receptors. Such conditions include, but are not limited to, leukemia, tumors involving nonhematopoietic cells, including small-cell carcinoma of the lung, melanoma, oat cell carcinoma, and inflammatory diseases, such as arthritis. The treatment of these diseases involves the use of a therapeutically effective amount of the conjugates formulated as a pharmaceutical composition in a physiologically acceptable carrier vehicle, such as phosphate buffered saline and saline.

Pharmaceutical carriers or vehicles suitable for administration of the conjugates provided herein include any such carriers known to those skilled in the art to be suitable for the particular mode of administration. In addition, the conjugates may be formulated as the sole pharmaceutically active ingredient in the composition or may be combined with other active ingredients.

Solutions or suspensions used for parenteral, intradermal, subcutaneous, or topical application can include any of the following components: a sterile diluent, such as water for injection, saline solution, fixed oil, polyethylene glycol, glycerine, propylene glycol or other synthetic solvent; antimicrobial agents, such as benzyl alcohol and methyl parabens; antioxidants, such as ascorbic acid and sodium bisulfite;

chelating agents, such as ethylenediaminetetraacetic acid (EDTA); buffers, such as acetates, citrates and phosphates; and agents for the adjustment of tonicity such as sodium chloride or dextrose. Parenteral preparations can be enclosed in ampules, disposable syringes or multiple dose vials made of glass, plastic or other suitable material.

If administered intravenously, suitable carriers include physiological saline or phosphate buffered saline (PBS), and solutions containing thickening agents, such as glucose, polyethylene glycol, and polypropylene glycol and mixtures thereof. Liposomal suspensions, including tissue-targeted liposomes, may also be suitable as pharmaceutically acceptable carriers. These may be prepared according to methods known to those skilled in the art. For example, liposome formulations may be prepared as described in U.S. Patent No. 4,522,811.

The conjugates may be formulated for local or topical application, such as for topical application to the skin in the form of gels, creams, and lotions and for application to the eye or for intracisternal or intraspinal application. Such solutions, particularly those intended for ophthalmic use, may be formulated as 0.01% - 10% isotonic solutions, pH about 5-7, with appropriate salts. The conjugates may be formulated as aeorsols for topical application, such as by inhalation (see, e.g., U.S. Patent Nos. 4,044,126, 4,414,209, and 4,364,923. Preferred compositions are formulated for oral or intravenous administration

The conjugates can be administered by any appropriate route, for example, orally, parenterally, intravenously, intradermally, subcutaneously, or topically, in liquid, semi-liquid or solid form and are formulated in a manner suitable for each route of administration. The conjugate is included in the pharmaceutically acceptable carrier in an amount sufficient to exert a therapeutically useful effect in the absence of serious toxic effects on the patient treated.

Therapeutically effective amounts of the conjugates may be administered as a single dosage or a series of dosages. Appropriate dosages may be administered intravenously, orally, subcutaneously, and by other methods of administration known in the art. It is understood that the precise method of treatment will vary with the pathological condition being treated.

The effective dosages and treatment regimes for individual pathological conditions may be determined empirically by testing procedures known in the art, including by extrapolation from in vivo or in vitro test data. See, for example, the determination of effective dosages for the FGF-SAP conjugate taught in International Patent Application Publication No. WO 92/04918 to THE SALK INSTITUTE FOR BIOLOGICAL STUDIES, which is based on U.S Patent Application Serial No. 07/585,319, filed on 19 September 1990.

Briefly, conjugates are tested in in vitro models of cancer, such as the assays described in the EXAMPLES, or other diseases. Once cytotoxicity is demonstrated, the conjugates are tested in in vivo models. For example, tumors may be implanted in immunodeficient nude mice to create a xenograft in the test animal. Such animals are treated with a range of dosages of the cytotoxic GM-CSF conjugate to assess toxicity and effectiveness. Appropriate dosage ranges can be estimated from lethal dose determination in the mice. Effective dosages may also be extrapolated from the results achieved in mice. For treatment, an effective dose would be one that ameliorates the treated condition without causing unacceptable side effects.

Typically, effective dosages will vary from approximately 0.1 μg to approximately 100 mg of pro-drug conjugate per kilogram of body weight per day. It is understood that effective dosages will vary with the particular toxic moiety of the conjugate. Due to the extremely low toxicity of the preferred GM-CSF-SAP conjugate, it appears that higher

dosages of this conjugate could be safely administered to mammals without serious toxic effects than would be possible for other types of conjugates.

Assays

The conjugates can also serve as tools for studies of the beneficial and deleterious functioning of GM-CSF in a mammal. For example, the mitotoxin provides a convenient assay for determining what cell types express GM-CSF receptors. Assays such as those described in Example 3 may be provided in which CTLL 2 and CTLL $\alpha\beta$-4/GM cells, for example, would be useful as controls for studying the effect of the GM-CSF-SAP on thymidine incorporation, or change in cell number, of a cell culture. A decrease in thymidine incorporation or cell number, for example, in response to an appropriate dosage of GM-CSF-SAP, would indicate the presence of receptors on the cells being tested.

The following examples are included for illustrative purposes only and are not intended to limit the scope of the invention.

EXAMPLE 1

A. Conjugation of GM-CSF with SAP

Saporin was purified from seeds of the plant Saponaria officinalis according to the method of Stirpe et al. (1983) Biochem J. 216:617-625. Recombinant human GM-CSF was obtained from Farmitalia Carlo Erba (Milan, Italy).

SAP was derivatized with N-succinimidyl-3(2-pyridyldithio) propionate (SPDP), (obtained from Pharmacia, Uppsala, Sweden) as described in Siena et al. (1988) Blood 72:756-765; see also, U.S. Patent No. 5,171,670 to Lappi et al.). GM-CSF was dissolved in water and dialyzed versus Dulbecco's phosphate-buffered saline without calcium and magnesium (PBX) and the volume adjusted to a concentration of 1 mg/ml. A three-fold molar excess of SPDP in 70 μL ethanol was added to 4.5 mg and incubated with agitation for 30 min. The reaction mixture

was dialyzed overnight against Dulbecco's PBS. As determined by following the manufacturer's instructions,there were 1.1 moles of pyridyl disulfide residue per mole of GM-CSF.

The blocking pyridine-2-thione group was removed from SAP by treatment with 50 μM dithiothreitol. A three-fold excess of thiolated SAP was added to pyridyl disulfide-GM-CSF (22.4 mg/3.6 mg). The reaction was monitored by following the increase in optical density at 343 nm. After 2.5 h, the reaction mixture buffer was changed to 30 mM TRIS, pH 8.0 by passing the solution through a G-25 column (Pharmacia, Uppsala, Sweden) and collecting the exclusion volume peak. The protein peak was divided into two parts which were chromatographed twice successively on a Mono Q 5/5 column (Pharmacia, Uppsalal, Sweden) equilibrated in 30 mM TRIS, pH 8.0 using a 30 min. gradient of 0 to 0.27 M sodium chloride in 30 mM TRIS, pH 8.0, and a flow rate of 0.5 ml/min. Fractions were collected and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (PhastSystem, Pharmacia). Fractions containing the GM-CSF-SAP conjugate were pooled, dialyzed against Dulbecco's PBS and the protein concentration in the dialysate determined. 1.8 mg of conjugate was recovered.

B. Western blotting

Identification of the conjugate was confirmed by the presence of SAP and GM-CSF in the 45,000 dalton band as detected by transfer of bands to nitrocellulose and Western blotting as described below.

A purified rabbit anti-human GM-CSF polyvalent antibody was purchased from Endogen, Boston, MA and used at a dilution of 1:500. The antiserum to SAP was raised in rabbits against the purified protein according to the standard methods (see, e.g., Lappi et al.(1985) Biochem. Biophys. Res. Comm. 129: 934) and was used at a dilution of 1:1000. Western blotting was accomplished by transfer of the electrophoresed protein to nitrocellulose using the PhastTransfer system (Pharmacia), as

described by the manufacturer. Horseradish peroxidase labeled anti-IgG was used as the second antibody as is known in the art.

EXAMPLE 2

Ribosome-Inactivating Activity of SAP Moiety of GM-CSF-SAP

The inhibition of protein synthesis resulting from the ribosome-inactivating activity of the SAP moiety of GM-CSF-SAP conjugate was compared to the activity of unconjugated SAP using in vitro assays that measure cell-free protein synthesis in a nuclease-treated rabbit reticulocyte lysate (Promega, Madison, WI). GM-CSF-SAP was treated immediately before assay with dithiothreitol (final concentration of 50 mM) for 1 h at 37°C to produce reduced GM-CSF-SAP. Samples were diluted in PBS and 5 μL of sample was added on ice to 35 μL of rabbit reticulocyte lysate and 10 μL of a reaction mixture containing 0.5 μg of Brome Mosaic Virus RNA, 1 mM amino acid mixture minus leucine, 5 μCi of tritiated leucine and 3 μL of water. Assay tubes were incubated 1 h in a 30°C water bath. The reaction was stopped by transferring the tubes to ice and 5 μL of the assay mixture was added in triplicate to 75 μL of 1 N sodium hydroxide, 2.5% hydrogen peroxide in the wells of a Millititer HA 96-well filtration plate (Millipore, Bedford, MA). When the red color had bleached from the samples, 300 μL of ice cold 25% trichloroacetic acid (TCA) were added to each well and the plate left on ice for another 30 min. The plate was placed on a Millipore vacuum holder attached to a vacuum source and the liquid vacuumed through. The wells were washed three times with 300 μL of ice cold 8% TCA. After drying, the filter paper circles were punched out of the 96-well plate and counted by liquid scintillation techniques.

Fig. 1 shows tritiated leucine incorporation in a rabbit reticulocyte lysate when exposed to GM-CSF-SAP, reduced GM-CSF-SAP, unconjugated SAP, and unconjugated GM-CSF. FIG. 1 shows that GM-

CSF-SAP must be reduced to restore the ribosome-inactivating activity of the SAP protein.

EXAMPLE 3

Cytotoxicity Studies

In the following described experiments, transfected and untransfected mouse T-cell CTLL cell lines were treated with GM-CSF-SAP, GM-CSF, SAP, and an equimolar mixture of GM-CSF and SAP.

CTLL 2 and CTLL $\alpha\beta$-4/GM were used in the experiments. CTLL $\alpha\beta$-4/GM cells are prepared by transfecting CTLL 2 cells with DNA encoding the α and β subunits of human GM-CSF (see, Kitamura et al. (1991) Proc. Natl. Acad. Sci. USA 88:5082-5086) and express recombinant human GM-CSF receptors. Procedures for transfection and conditions for maintenance of cell lines are known to those of skill in the art. The procedures used in the experiments herein are described, for example, in Kitamura et al. (1991) Proc. Natl. Acad. Sci. USA 88:5082-5086.

CTLL 2 cells were cultured in the following medium: RPMI-1640, 10% fetal bovine serum, 50 μM 2-mercaptoethanol and mouse interleukin-2, 100 units/mL. CTLL$\alpha\beta$-4/GM cells were cultured in the following medium: RPMI-1640, 10% fetal calf serum, 50 μM 2-mercaptoethanol, and hGM-CSF, 1 ng/mL.

Aliquots of cells in 100 μL of growth medium were seeded to the wells of a 96-well plate at 1000 cells per well. Eighteen hours later, samples diluted in growth medium were added in quadruplicate. After a 72-h incubation tritiated thymidine (50 nCi/well) was added to the wells for 6 h and the plates were frozen at -80°C overnight. The plates were then thawed and well contents collected with a Skatron cell harvester onto glass fiber filter sheets and radioactive incorporation

determined by scintillation counting. The counts in treated wells were normalized to control wells to which only medium had been added.

Fig. 2a shows the effects of exposure to GM-CSF-SAP, GM-CSF, SAP and an equimolar mixture of GM-CSF and SAP. In the experiment with CTLL$\alpha\beta$-4/GM cells as shown in Figure 2a, there were 18,222 ± 1,340 CPM per well for the control group; whereas, cells treated with 10 nM GM-CSF-SAP, there were 242 ± 40 CPM.

To ascertain the effects of GM-CSF-SAP, GM-CSF, SAP, and an equimolar mixture of GM-CSF and SAP on cell number of transfected CTLL$\alpha\beta$-4/GM cells, 1 mL aliquots of cells were seeded in wells of 24-well plates at concentrations of 20,000 cells/mL of growth medium. Thirty minutes later, 10 μL of each of the samples, diluted in growth medium to 100 times the final assay concentration, were added in triplicate. After a 72-h incubation, the cells and media were removed to 9 mL of isotonic diluent and counted with a Coulter cell counter (Coulter Electronics, Hialeah, FL). The cell number in treated wells was compared with control wells to which 10 μL of medium only had been added.

The results are shown in Fig. 2b. For the experiment with CTLL$\alpha\beta$-4/GM cells in Figure 2b, there were 148,658 ± 7,573 cells per well (N = 18) in the control group. Treatment with 10 nM GM-CSF-SAP, resulted in 29,287 ± 562 cells per well.

Fig. 2c shows the effect of the conjugate GM-CSF-SAP on tritiated thymidine uptake by transfected CTLL $\alpha\beta$-4/GM cells and untransfected CTLL 2 cells. The procedure followed for this experiment is the same as that outlined above for the experiment with results shown in Fig. 2a.

In comparing Fig. 2a with Fig. 2b, it can be seen that while thymidine incorporation drops to nearly zero, there still remains almost 20% of control cell count in the cell count assay. This is probably an artifact due to dead cells and debris that remain in the wells. The results in Fig. 2a show that the residual cells are unable to incorporate

thymidine. Fig. 2a also shows inhibition of thymidine incorporation at high doses of the non-conjugated mixture of GM-CSF and SAP. This is not seen in Fig. 2b.

The approximately five-fold increase in cytotoxicity of the mixture compared to SAP may indicate an effect on the membrane by GM-CSF.

EXAMPLE 4

Inhibition of cytotoxicity of GM-CSF-SAP by GM-CSF and other growth factors

For the following two experiments, the IL-2, GM-CSF dependent CTLL cell line AIC2Bα-3 was employed. This cell line was transfected with DNA encoding human α subunit and DNA that encodes the mouse AIC2B protein. Mouse AIC2B protein is 91% homologous to the protein encoded by the mouse AIC2A gene, which encodes the β low affinity subunit of the mouse IL-3 receptor. IL-2 (Bachem, Inc., Torrance, CA) was used as the growth factor for these cells. CTLL 2 and CTLL AIC2$\alpha\beta$-3 cells were cultured in the following medium: RPMI-1640, 10% fetal bovine serum, 50 μM 2-mercaptoethanol and mouse interleukin-2, 100 units/mL following the procedure of Kitamura et al. (1991) Proc. Natl. Acad. Sci. USA 88:5082-5086.

In the first set of experiments, the results of which are given in Fig. 3a, the CTLL cell line AIC2Bα-3 was treated with 100 pM (0.1 nM) GM-CSF-SAP in addition to increasing concentrations of GM-CSF growth factor. The first column shows incorporation of the cells with no addition of either GM-CSF or GM-CSF-SAP. Standard deviations are indicated and are typical of all thymidine incorporation experiments. The second column shows the effect of 0.1 nM GM-CSF-SAP alone. The remaining columns show the effect of 0.1 nM GM-CSF-SAP on thymidine incorporation in the presence of increasing concentrations of GM-CSF.

In the second set of tests, shown in Fig. 3b, GM-CSF receptor-bearing cells were treated with 200 pM GM-CSF-SAP alone (first column), or with 100-fold excess of growth factors including GM-CSF as

indicated in Fig. 3b. In these experiments, the CTLL cell line AIC2Bα-3 was treated with 200 pM GM-CSF-SAP and 20 nM of the commercially available growth factors hGM-CSF, basic FGF, EGF, IGF-1, IGF-2, and TGF-α. The conjugate and growth factors were added simultaneously.

Aliquots of cells in 100 μl of growth medium (RPMI-1640, 10% fetal bovine serum, 50 μM 2 mercaptoethanol and mouse interleukin-2, 100 units/ml) were seeded in wells of a 96-well plate at 1000 cells per well. Eighteen hours later, samples containing GM-CSF, GM-CSF-SAP and other growth factors diluted in growth medium were added in quadruplicate. After a 24-h incubation, ^{3}H-thymidine (50 nCi/well) was added to the wells for 6 hours and the plates were frozen overnight. The plates were then thawed and well contents were collected with a Skatron cell harvester onto glass fiber filter sheets and radioactivity incorporation determined by scintillation counting. As indicated, other growth factors were added simultaneously with mitotoxin to test for specificity. For the tests shown in Figure 3b, the control CPM were 3,463 $\pm$ 489.

Fig. 3a shows that there is a dose-dependent inhibition of the cytotoxicity. An equimolar concentration of GM-CSF-SAP and GM-CSF results in no significant inhibition. With a ten-fold, hundred-fold, and thousand-fold excess of GM-CSF, there is an increasing inhibition of cytotoxicity. This is consistent with a competitive inhibition of GM-CSF-SAP by GM-CSF for the GM-CSF receptor and demonstrates that the mitotoxin acts through the GM-CSF receptor.

Fig. 3b shows that only GM-CSF growth factor inhibits the cytotoxic effect of GM-CSF-SAP. A 100-fold excess of other growth factors does not reverse the inhibition of thymidine incorporation. This is further evidence that GM-CSF-SAP acts through the GM-CSF receptor.

Since modifications will be apparent to those of skill in this art, it is intended that this invention be limited only by the scope of the appended claims.

WHAT IS CLAIMED IS:

1. A cytotoxic conjugate, comprising a hematopoietic growth factor granulocyte-macrophage colony-stimulating factor (GM-CSF) coupled to a type 1 ribosome-inactivating protein.

2. The conjugate of claim 1 that exhibits reduced ribosome-inactivating activity compared to the unconjugated ribosome-inactivating protein in cell free assays but is cytotoxic to GM-CSF receptor bearing cells.

3. A cytotoxic conjugate, comprising a hematopoietic growth factor granulocyte-macrophage colony-stimulating factor (GM-CSF) coupled to the ribosome-inactivating protein saporin.

4. The conjugate of claim 3, wherein saporin is coupled by disulfide bonding to the GM-CSF.

5. The conjugate of any of claims 1-4, wherein the granulocyte-macrophage colony-stimulating factor (GM-CSF) is human granulocyte-macrophage colony-stimulating factor.

6. The conjugate of any of claims 1-4 that is reactive with human GM-CSF receptor on mammalian cell surfaces.

7. The conjugate of claim 3, wherein GM-CSF and SAP are present in a molar ratio of about 1 to 1.

8. A method of inhibiting the proliferation of cells expressing GM-CSF receptors, comprising administering the conjugate of claim 1 or claim 2 to cells bearing GM-CSF receptors.

9. A method of treating pathological conditions involving GM-CSF expression comprising administering a therapeutically effective dosage of the conjugate of claim 1 or claim 2.

10. The method of claim 9, wherein condition is an inflammatory disease or a cancer.

11. The method of claim 9, wherein condition is arthritis.

12. The method of claim 9, wherein condition is leukemia.

13. The method of claim 9 wherein the condition is one or more solid tumors.

14. The method of claim 13, wherein the condition is malignant melanoma.

15. An assay for detecting GM-CSF receptors on the cell surfaces of primary or secondary cell cultures, comprising contacting the cell cultures with a conjugate of claim 1 or claim 2 and measuring he thymidine incorporation by the cell cultures or cell number, wherein a reduction in incorporation or cell number following contacting with the conjugate indicates that the cells bear GM-CSF receptors.

16. A pharmaceutical, comprising an effective amount of the conjugate of claim 1 or claim 2 and a physiologically acceptable carrier, wherein the amont is effective for single dosage delivery.

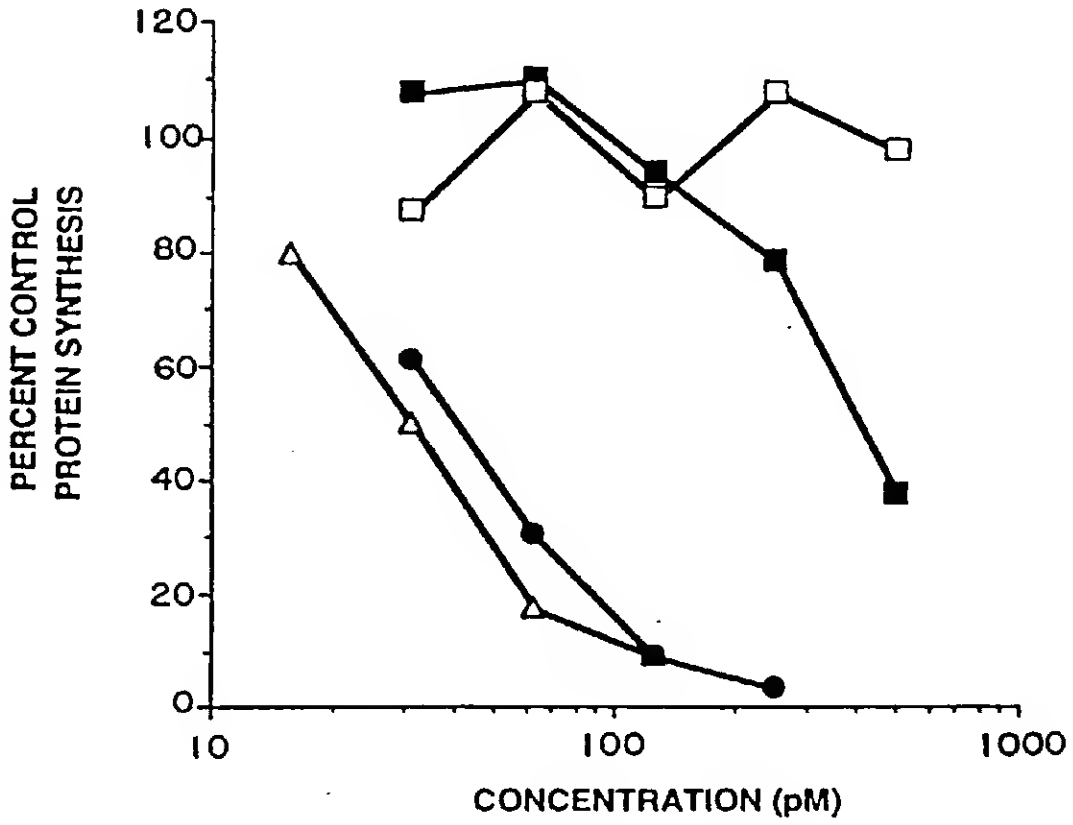

FIG. 1

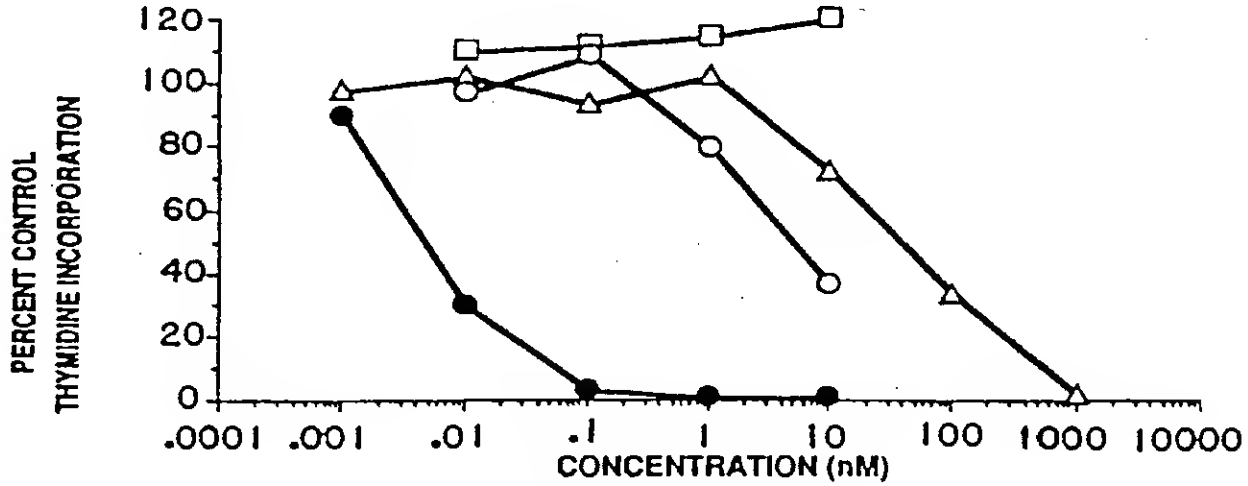

FIG. 2A

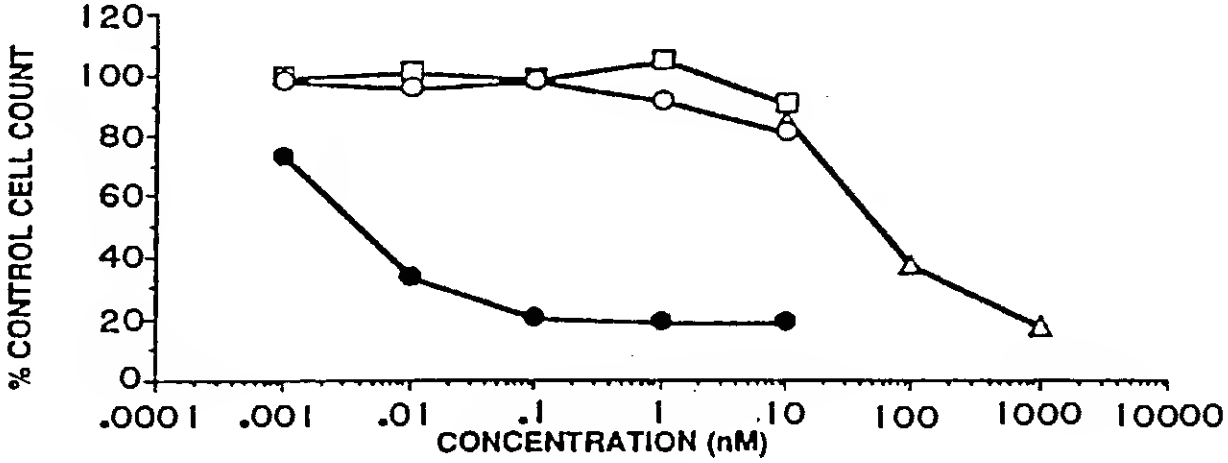

FIG. 2B

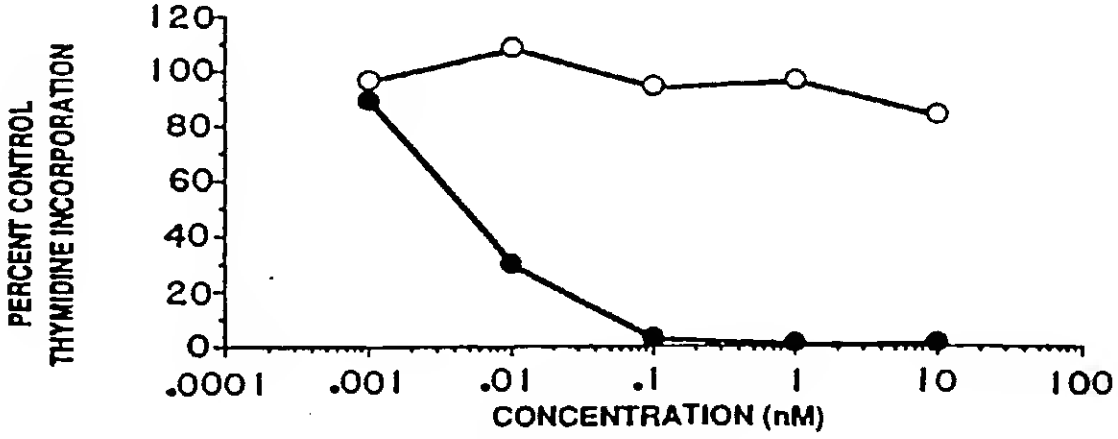

FIG. 2C

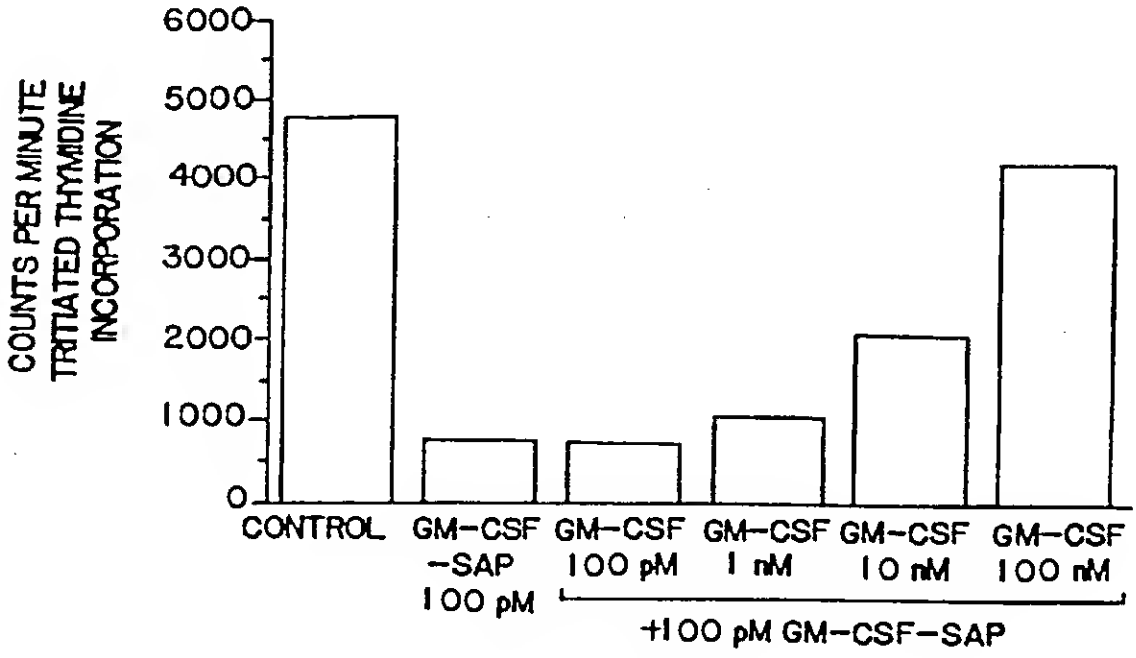

FIG. 3A

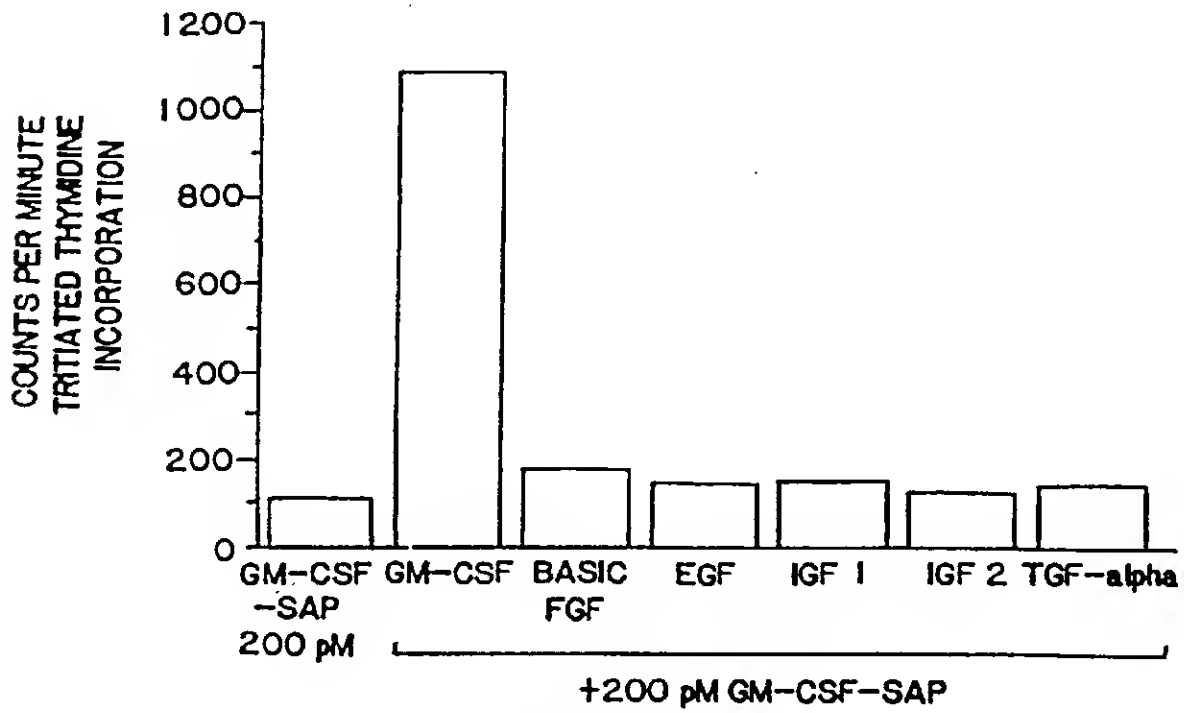

FIG. 3B

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 93/05574

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) *

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[5]: A 61 K 37/36, G 01 N 33/68

II. FIELDS SEARCHED

Minimum Documentation Searched †

Classification System	Classification Symbols
IPC[5]	A 61 K, G 01 N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched ‡

III. DOCUMENTS CONSIDERED TO BE RELEVANT*

Category *	Citation of Document, ** with indication, where appropriate, of the relevant passages ††	Relevant to Claim No. ††
A	AU, B, 23 587/88 (SANDOZ LTD.; INSTITUTO NAZIONALE PER LO STUDIO E LA CURA DEI TUMORI) 12 April 1990 (12.04.90), claim 1. --	1
A	BLOOD, vol. 77, no.6, 15 March 1991, New York J.C. GASSON "Molecular Physiology of Granulocyte--Macrophage Colony--Stimulating Factor", pages 1131-1145; the whole document (cited in the application). --	1
A	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA, vol. 85, no. 23, December 1988, Baltimore, USA	1

* Special categories of cited documents: ††

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
06 October 1993	05 [illegible]
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	WOLF e.h.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, ** with indication, where appropriate, of the relevant passages	Relevant to Claim No.
	S. DEDHAR et al. "Human granulocyte-macrophage colony-stimulating factor is a growth factor active on a variety of cell types of nonhemopoietic origin", pages 9253-9257; the whole document (cited in the application). ----	

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 93/05574

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 9-14
because they relate to subject matter not required to be searched by this Authority, namely:

According to rule 39.1(iv) PCT

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

ANHANG

zum internationalen Recherchenbericht über die internationale Patentanmeldung Nr.

ANNEX

to the International Search Report to the International Patent Application No.

ANNEXE

au rapport de recherche international relatif à la demande de brevet international n°

PCT/US 93/05574 SAE 76146

In diesem Anhang sind die Mitglieder der Patentfamilien der im obengenannten internationalen Recherchenbericht angeführten Patentdokumente angegeben. Diese Angaben dienen nur zur Unterrichtung und erfolgen ohne Gewähr.

This Annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The Office is in no way liable for these particulars which are given merely for the purpose of information.

La présente annexe indique les membres de la famille de brevets relatifs aux documents de brevets cités dans le rapport de recherche international visée ci-dessus. Les renseignements fournis sont donnés à titre indicatif et n'engagent pas la responsibilité de l'Office.

Im Recherchenbericht angeführtes Patentdokument Patent document cited in search report Document de brevet cité dans le rapport de recherche	Datum der Veröffentlichung Publication date Date de publication	Mitglied(er) der Patentfamilie Patent family member(s) Membre(s) de la famille de brevets	Datum der Veröffentlichung Publication date Date de publication
AU A 23587		AU A1 23587/88	12-04-90
		AU B2 618283	19-12-91